J. Clin. Chem. Clin. Biochem.
Vol. 25, 1987, pp. 101–106

Radioimmunoassay of 5-Hydroxy-3-indole Acetic Acid

By *B. Manz, H. J. Grill, O. Belovsky, I. Kleinböhl, A. Heubner* and *K. Pollow*

Abteilung für Experimentelle Endokrinologie der Johannes-Gutenberg-Universität Mainz

(Received May 20/August 25, 1986)

Summary: A direct radioimmunoassay of the methyl ester of urinary and serum 5-hydroxy-3-indole acetic acid is described. The antiserum, raised in a rabbit against a conjugate of bovine serum albumin with 5-hydroxytryptamine hemisuccinamide, contained two antigenic fractions, one binding N-acyl 5-hydroxytryptamine, and the other binding methyl ester of 5-hydroxy-3-indole acetic acid, and N-acyl 5-hydroxytryptamine. The N-acyl 5-hydroxytryptamine binding fraction was removed by affinity chromatography on a N-acyl 5-hydroxytryptamine agarose gel in the presence of excess methyl ester of 5-hydroxy-3-indole acetic acid. The antibody methyl ester of 5-hydroxy-3-indole acetic acid complexes were dissociated and this affinity-purified antiserum was used in all experiments. Polyethylene glycol in combination with goat anti-rabbit IgG was used to separate bound and unbound ^{125}I-labeled *Bolton-Hunter* reagent- 5-hydroxytryptamine conjugate. Sample preparation (esterification of 5-hydroxy-3-indole acetic acid to its methyl ester) was performed with trimethylsilyldiazomethane in dioxane. In the analysis of urine, the reagents used in the methylation served as diluents, contributing to the final dilution of 1 : 1100. In the analysis of serum, a deproteination step (ethanol precipitation) prior to methylation was necessary to obtain reproducible results. The methylated 5-hydroxy-3-indole acetic acid was then extracted with ethyl acetate and the extract redissolved in assay buffer. The minimal detectable concentration of methyl ester of 5-hydroxy-3-indole acetic acid was 1.1 µmol/l (0.21 mg/l 5-hydroxy-3-indole acetic acid) urine or 100 fmol/tube. The intra-assay precision (CV) for urine samples was 6.4% (n = 20) at a level of 22 µmol/l, and 9.6% (n = 20) at a level of 230 µmol/l. The inter-assay CV was 11% at a level of 230 µmol/l. The only substance cross-reacting with the antibody was N-acetylserotonin which was not detectable in urine when the esterification step was omitted. To validate the clinical usefulness of this assay, a comparison with the commercially available BioRad® column assay was performed. Both radioimmunoassay and fluorescence determination accurately identified two patients with known carcinoid syndrome. A correlation of r = 0.817 was demonstrated between the two assays in a comparison of normal and pathological urines. A simultaneous determination of serotonin and its metabolite 5-hydroxy-3-indole acetic acid in normal and pathological sera showed that both parameters were raised in carcinoid syndrome.

Introduction

Carcinoid tumours generally secrete various amounts of indoles. Many of them secrete other substances such as bradykinin, corticotropin, and histamine (1), but carcinoid syndrome is generally characterized by an increased urinary excretion of 5-hydroxy-3-indole acetic acid, the end product of serotonin metabolism (2). Traditionally, this compound is assayed by diazotization with nitrosonaphthol to form a purple colour (3). However, it is well documented that many other substances present in the urine interfere with this reaction to give false-positive results (4). Attempts were made to overcome this problem by a combination of ion exchange chromatography and fluorometry (BioRad® column test). This method, however, lacks sensitivity and is time consuming. Recently, high performance liquid chromatographic analyses of 5-hydroxy-3-indole acetic acid with fluorometry in the ultraviolet region of the spectrum (5) or electrochemical detection (6) have been described. Both methods require solvent extraction because of the numerous interfering compounds present in urine.

Although the induction of antibodies against 5-hydroxy-3-indole acetic acid has been reported, the low titers of anti-5-hydroxy-3-indole acetic acid antisera (7, 8) or the complexity of the assay procedure (9) has strictly limited their applicability. Based on the previously described radioimmunoassay of N-acetylated 5-hydroxytryptamine (10), it was therefore decided to establish a radioimmunoassay for the determination of the methyl ester of 5-hydroxy-3-indole acetic acid in urine and serum, using a structurally related [^{125}I]N-acyl analogue of 5-hydroxytryptamine. The specific conversion of 5-hydroxy-3-indole acetic acid into its methyl ester by trimethylsilyldiazomethane (11) and the use of a ^{125}I analogue as radioactive ligand in combination with the double antibody technique facilitate the determination of 5-hydroxy-3-indole acetic acid in a routine laboratory.

Materials and Methods

Reagents

Chemicals

N-Succinimidyl-3-(4-hydroxy-5(3)-[^{125}I]iodophenyl)propionate (^{125}I-labeled *Bolton-Hunter* reagent, 74 TBq/mmol) and 5-hydroxy[1,2-^{3}H(N)]tryptamine: creatinine sulphate (1.1 TBq/mmol) were from New England Nuclear Corp. (Dreieich, F. R. G.). Bovine serum albumin was from Behring Institut (Marburg, F. R. G.). 5-Hydroxy-3-indole acetic acid and its analogues were from Sigma (München, F. R. G.) and Aldrich (Nettetal, F. R. G.). Analytical grade chemicals and glass-distilled water were used throughout.

Buffers

Buffers used are identified by the following abbreviations: buffer A (50 mmol/l potassium phosphate, pH 7.0, containing 3 mmol/l sodium azide and 1 g/l ascorbic acid); buffer B (buffer A supplemented with 1 g/l gelatine).

Tracer solution

Appropriate amounts of the ^{125}I-labeled *Bolton-Hunter* reagent-5-hydroxytryptamine conjugate diluted with buffer B to a final radioactivity of 10 MBq/l.

Antiserum solution

The affinity purified antiserum was tested at different dilutions in buffer A. The dilution resulting in 25% binding of the tracer under assay conditions was used.

Precipitating antiserum reagent

The precipitating antiserum (goat anti-rabbit IgG, polyethylene glycol 4000, sodium azide as preservative) was purchased from DDV Diagnostika (Marburg, F. R. G.).

Preparation of immunogen and immunisation

The immunogen used consisted of 5-hydroxytryptamine hemisuccinamide bound to bovine serum albumin as described (9, 11). Rabbits were immunized with an initial dorsal injection of an emulsion of 0.5 ml (1 mg) of immunogen and 0.5 ml of complete *Freund*'s adjuvant. Booster injections were given in the same way at 4 week intervals except that incomplete *Freund*'s adjuvant was used.

Iodination of 5-hydroxytryptamine (^{125}I-labeled *Bolton-Hunter* reagent-5-hydroxytryptamine conjugate). Five mg of 5-hydroxytryptamine were dissolved in 500 µl dry pyridine, 10 µl aliquots of this solution were mixed with appropriate amounts of ^{125}I-labeled *Bolton-Hunter* reagent and the conjugate purified by reversed phase high performance liquid chromatography as previously described (10).

Purification of anti-methyl ester of 5-hydroxy-3-indole acetic acid antibodies (affinity purified antiserum)

I. Preparation of 5-hydroxytryptamine affinity gel
Commercially available Affigel 10 (BioRad, Richmond, USA), a N-hydroxysuccinamide active ester derivative of cross-linked agarose beads, was used as activated support. 5-Hydroxytryptamine hydrochloride (500 mg plus 1.8 MBq[^{3}H]5-hydroxytryptamine; final specific activity 0.66 GBq/mol) was dissolved in 20 ml of 1 mol/l NaOH and the free amine was extracted three times with 50 ml of $CHCl_3$. The organic solvent was evaporated in vacuo and the remaining oil dissolved in ethanol. The final concentration of 5-hydroxytryptamine was 1 mol/l. Affigel 10 was used without further purification. 5-Hydroxytryptamine solution (2 ml) was added to a suspension of 25 ml gel in 25 ml ethanol and the reaction mixture was gently shaken overnight at 4 °C. The gel was recovered by filtration and washed with 2 l of buffer A. Based on the specific activity of the affinity gel (determined after solubilization of 0.5 ml of gel suspension with 30% H_2O_2), approximately 5 mmol 5-hydroxytryptamine were covalently bound per litre of gel. In the presence of 1 g/l ascorbic acid the gel was stable for several months at 4 °C.

II. Affinity chromatography
Affinity chromatography was performed as previously described (12). Briefly, 10 ml of antiserum were diluted with 90 ml of buffer A and incubated with 1 mg/l of methyl ester of 5-hydroxy-3-indole acetic acid for 2 h at 37 °C. Affinity gel (1 g) was added and the mixture rotated for 18 h at 4 °C. The suspension was poured into a sintered glass funnel and the filtrate collected. In order to remove excess methyl ester of 5-hydroxy-3-indole acetic acid, the eluate was incubated for 5 h with 5 ml of protein A sepharose (Pharmacia, Sweden), followed by a short washing step in a sintered glass funnel. To release the protein A-bound IgG, the affinity gel was eluted with two volumes of 0.1 mol/l acetic acid. The eluate was collected directly into 0.5 mol/l disodium phosphate to neutralize the pH (13), 10 ml of rabbit preimmune serum were added, and the solution was dialysed against phosphate buffered saline, then lyophilized. The lyophilized anti-methyl ester of 5-hydroxy-3-indole acetic acid antibody-enriched serum (affinity purified antiserum) was dissolved in 10 ml of distilled water and stored at −20 °C until use.

Sample preparation

Urine

Aliquots of acidified 24 h urine were stored in the dark at about 4 °C, or frozen if analysis was more than 48 h later. Lyophilized quality control samples (Lypho Check®, Anaheim, California, USA) were reconstituted with 0.1 mol/l HCl and stored frozen at −20 °C.

Conversion of 5-hydroxy-3-indole acetic acid to its methyl ester

Urine specimens (100 µl) were diluted with 2 ml of methanol. This diluted urine (100 µl) was added to a conical glass tube, followed by 25 µl of a 18% solution of trimethylsilyldiazome-

thane in dioxan (Ventron, Karlsruhe, F. R. G.). Excess reagent was destroyed by addition of 25 µl of 0.1 mol/l HCl. The methylated probe was then further diluted with 5 ml of buffer B, and 100 µl aliquots were withdrawn for radioimmunoassay.

Serum

To avoid clotted fibrin, blood samples were collected by use of Monovette® syringes (Sarstedt, Nümbrecht, F. R. G. or similar products), and centrifuged at 1500 g. The serum was separated and frozen at −20 °C before analysis (within two weeks). All samples were taken only during normal waking hours, but at no specific time.

5-Hydroxy-3-indole acetic acid extraction and conversion to its methyl ester

Serum (200 µl) and ethanol (400 µl) were added to a glass tube and carefully vortexed. The mixture was kept for 30 min at 4 °C to complete the precipitation of proteins, then centrifuged at 2000 g. Clear supernatant (400 µl) was decanted into a glass tube. Methanol (200 µl) and 18% trimethylsilyldiazomethane in dioxan (50 µl) were added and the mixture kept for 10 min at room temperature. HCl (0.1 mol/l, 50 µl) and water (2 ml) were added and the methylated 5-hydroxy-3-indole acetic acid extracted with 4 ml of ethyl acetate. An aliquot of the organic extract (3 ml) was withdrawn and the solvent removed by evaporation under a gentle stream of nitrogen. Assay buffer (250 µl) was added and the 2.5-fold diluted extract stored at 0−4 °C until use (no longer than 1 month).

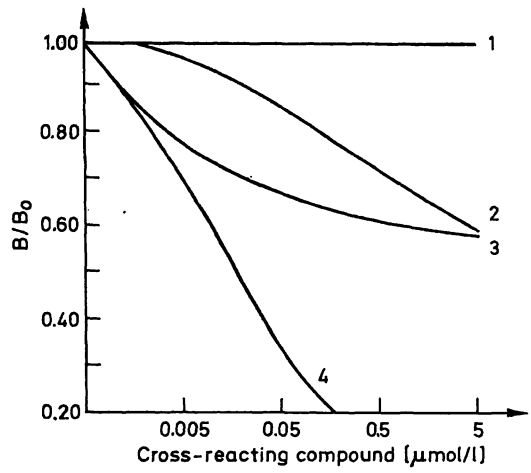

Fig. 1. Inhibition by 5-hydroxytryptamine analogues of binding of ^{125}I-labeled *Bolton-Hunter*-5-hydroxytryptamine conjugate by antibody.
Aliquots of diluted antiserum (100 µl) were incubated in triplicate with tracer solution (100 µl, 1 kBq/tube) and increasing concentrations (0−5 µmol/l) of the various competitors. Antibody-bound radioactivity was determined by use of the double antibody technique.
The numbers represent: (1) 5-hydroxy-3-indole acetic acid; (2) 5-hydroxytryptophol; (3) methyl ester of 5-hydroxy-3-indole acetic acid; (4) 5-hydroxytryptamine (non-radioactive *Bolton-Hunter*-5-hydroxytryptamine conjugate, see table 2).

Radioimmunoassay procedure

The methylated urine or serum preparation (100 µl), tracer solution (100 µl, 1 kBq/tube) and diluted affinity purified antiserum (100 µl, final dilution 1 : 600) were added to a polystyrene tube and incubated overnight at 4 °C. One ml of precipitating antibody was added, the mixture incubated for 1 h at 4 °C and centrifuged. The supernatant was aspirated and the pelleted radioactivity determined in a gamma-counter. Each series consisted of a standard curve (0.0052−0.52 µmol/l methyl ester of 5-hydroxy-3-indole acetic acid), blanks for the determination of unspecific binding, quality control samples and the samples to be assayed. The serum values were corrected for dilution during sample preparation by multiplying by 2.5.

Results

Antibody preparation

Approximately 50 percent of the previously reported polyclonal anti N-acetylserotonin antibodies are able to bind methyl ester of 5-hydroxy-3-indole acetic acid with high affinity (fig. 1) whereas all antibodies are able to bind N-acyl analogues of 5-hydroxytryptamine (10). This property was used to separate the methyl ester of 5-hydroxy-3-indole acetic acid binding fraction from the exclusively N-acyl 5-hydroxytryptamine binding fraction by affinity chromatography. As the affinity gel consisted of 5-hydroxytryptamine coupled to activated carboxyl groups of cross-linked agarose, only the N-acyl 5-hydroxytryptamine binding fraction was retained on the gel in the presence of excess methyl ester of 5-hydroxy-3-indole acetic acid.

The methyl ester of 5-hydroxy-3-indole acetic acid-masked antibody fraction was isolated by a second affinity chromatography step. As protein A sepharose specifically binds to the Fc region of the IgG molecule, a dialysis step was necessary to isolate methyl ester of 5-hydroxy-3-indole acetic acid-free antibodies. The affinity purified antibodies were supplemented with preimmune serum to enhance the precipitation efficiency of the second anti-rabbit IgG antibodies during the radioimmunoassay procedure.

Esterification of 5-hydroxy-3-indole acetic acid to its methyl ester

The esterification of indole acids with trimethylsilyldiazomethane and methanol proceeds instantaneously when an excess of the reagent is used, and the reaction can be easily monitored by the disappearance of the yellow colour (tab. 1). For diluted urine specimens, most of the reagent was needed to destroy the hydrochloric acid which was added to the urine to stabilize the 5-hydroxy-3-indole acetic acid. Saponification of the ester bond under assay conditions was not observed.

Tab. 1. Esterification of some carboxylic acids with trimethylsilyldiazomethane.

	R–COOH	Isolated yield of $R-C(=O)-O-CH_3$
1	5-Hydroxy-3-indole acetic acid	Quantitative
2	Indole-3-acetic acid	Quantitative
3	Indole-3-pyruvic acid	Quantitative
4	3-Indole acrylic acid	Quantitative
5	*D,L*-Vanillylmandelic acid	Quantitative
6	Homovanillic acid	Quantitative

A general experimental procedure for the preparation of methyl esters is as follows: Trimethylsilyldiazomethane (0.13 mmol) in 100 µl of dioxan was added to a stirred solution of the carboxylic acid (0.1 mmol) in methanol (5 ml) at room temperature. The mixture was concentrated and the purity was checked by thin-layer chromatography on Merck F-254® (Merck, Darmstadt, F. R. G.) precoated silica gel plates in solvent systems chloroform/methanol (9 + 1, by vol.), ethyl acetate/ethanol/NH_3 (5 + 5 + 1), and *n*-butanol/acetic acid/H_2O (12 + 3 + 4, by vol.).

Standard curve

A typical standard curve is shown in figure 2. The standard curve is sigmoid over the range of 0.47–47 pmol/tube. As the sample preparation includes a 1100-fold dilution of urine specimen, the standard curve actually covers the range of 5.2–520 µmol/l urine.

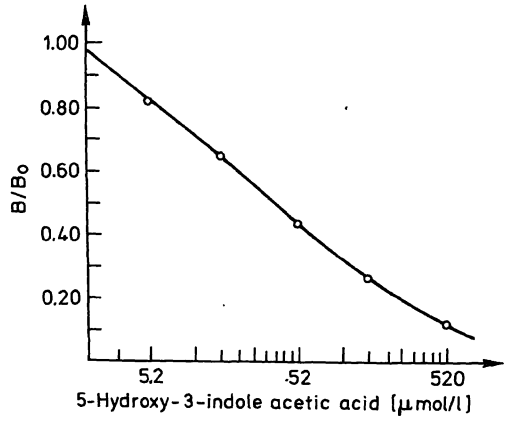

Fig. 2. Typical standard curve for the radioimmunoassay of methyl ester of 5-hydroxy-3-indole acetic acid.
The abscissa represents the concentrations of methyl ester of 5-hydroxy-3-indole acetic acid in the standards corrected for the 1100-fold dilution of urine during sample preparation.

Minimum detectable concentration

The within-day CV of the zero-dose standard is < 2%. Therefore, the minimal detectable concentration is the concentration at which B/B_0 is approximately 0.96 (1–2 CV), or 1.1 µmol/l urine (0.1 pmol/tube).

Specificity

The specificity of antibody for methyl ester of 5-hydroxy-3-indole acetic acid is shown in table 2. The data indicate that in addition to acylation of the amino group of 5-hydroxytryptamine (10), esterification of the carboxy group of 5-hydroxy-3-indole acetic acid is also a prerequisite for the specific recognition of the 5-hydroxyindole nucleus by this antibody.

Tab. 2. Specificity of anti-methyl ester of 5-hydroxy-3-indole acetic acid.

Compound	Cross reaction [%]*)
5-Hydroxy-3-indole acetic acid methyl ester	100
5-Hydroxy-3-indole acetic acid	< 0.1
Indole-3-acrylic acid	< 0.01
Indole-3-acrylic acid methyl ester	< 4
Indole-3-pyruvic acid	< 0.01
Indole-3-pyruvic acid methyl ester	< 0.4
Indole-3-acetic acid	< 0.1
Indole-3-acetic acid methyl ester	< 2
5-Hydroxytryptamine	< 0.1
N-[3-(4-hydroxy-3(5)-iodophenyl)-propionyl]-5-hydroxytryptamine	900
N-Acetylserotonin	800
5-Hydroxy-*D,L*-tryptophan	< 0.1
5-Hydroxy-*D,L*-tryptophan methyl ester	< 0.1
5-Hydroxytryptophol	< 3
Tryptamine	< 0.1
Melatonin	< 1
5-Methoxytryptophol	< 0.01
5-Methoxytryptamine	< 0.01
D,L-Vanillylmandelic acid	< 0.01
D,L-Vanillylmandelic acid methyl ester	< 0.01

*) The percent cross reaction is determined by dividing the mass of methyl ester of 5-hydroxy-3-indole acetic acid at 0.5 B/B_0 by the mass of the cross-reacting compound at 0.5 B/B_0 and multiplying by 100.

The specificity of antibody for methyl ester of 5-hydroxy-3-indole acetic acid in methylated serum extracts is shown in figure 3. Reversed phase high performance liquid chromatography reveals only one peak detectable by radioimmunoassay. The retention time of this peak is identical with that of authentic methyl ester of 5-hydroxy-3-indole acetic acid.

Recovery

Urine

Twenty different urine specimens were tested. Aliquots were enriched with 26 and 52 µmol/l 5-hydroxy-3-indole acetic acid, and the differences between enriched and normal urines determined by radioimmunoassay. Analytical recoveries were 99% (SD 5.4%) and 101% (SD 7.4%), respectively.

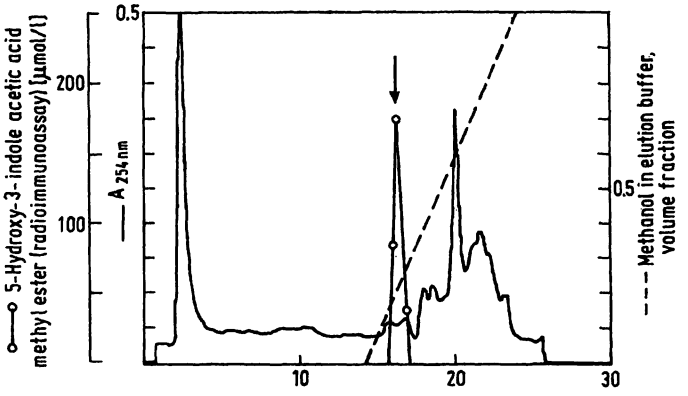

Fig. 3. High performance liquid chromatography of a human serum extract (carcinoid syndrome).
Human serum (1 ml) and ethanol (2 ml) were mixed, the precipitated proteins removed by centrifugation and the supernatant methylated and extracted with ethyl acetate as described in Material and Methods. In contrast to the radioimmunoassay procedure, the methylated extract was redissolved in water/methanol (9 + 1, by vol.) and submitted to reversed phase high performance liquid chromatography on a PEP RPC column (Pharmacia, Freiburg, F. R. G.). Fractions of 1 ml were collected for the determination of methyl ester of 5-hydroxy-3-indole acetic acid by radioimmunoassay. The arrow (↓) indicates the retention time of authentic methyl ester of 5-hydroxy-3-indole acetic acid.

Serum

Pooled human serum was enriched with 0.52 and 2.6 μmol/l 5-hydroxy-3-indole acetic acid, and the differences between enriched and pooled sera determined by radioimmunoassay. Analytical recovery was > 85%.

Precision

Twenty urine specimens from a normal volunteer and Lypocheck® Control Urine II C (Lot. No. 00200) were stored in the dark at −20 °C and used to estimate the intra- and inter-assay precision. The intra-assay CV for 22 μmol/l (normal volunteer) and 230 μmol/l (control urine) was 6.4% and 9.6%, respectively (29 consecutive determinations). The inter-assay CV was 11% for 230 μmol/l.

Fluorescence assay of urinary 5-hydroxy-3-indole acetic acid

A comparison of the BioRad® column test (a combination of ion exchange chromatography and chemical transformation of the partly purified acid to a fluorochrome with subsequent fluorescence measurement) and the radioimmunoassay showed the following correlation between both methods (tab. 3). Linear regression analysis of the data: r = 0.842, y-intercept = 7.3, slope = 0.817.

Tab. 3. Comparison of 5-hydroxy-3-indole acetic acid radioimmunoassay and the BioRad® column test[a]).

Sample No.	BioRad (μmol/l)	RIA	Sample No.	BioRad (μmol/l)	RIA
1	67.5	57.6	16	0.52	24.6
2	62.8	62.3	17	20.9	28.7
3	18.8	25.1	18	20.9	12
4	18.8	15.7	19	9.4	5.8
5	21.4	17.2	20	16.2	15.7
6	21.4	13.6	21	16.2	23.5
7	14.6	14.9	22	14.6	19.3
8	14.6	20.4	23	14.6	37.6
9	18.8	29.8	24	[off curve	581[b])]
10	18.8	20.9	25	[off curve	371[b])]
11	4.7	6.3	26	10.4	13.6
12	4.7	13.6	27	28.2	35.1
13	20.9	25.1	28	29.3	42.9
14	5.2	2.8	29	35.6	45
15	28.7	24.6	30	[214	230[c])]

y = 7.30 + 0.817x; r = 0.842

[a]) Fluorescence measurement
[b]) Patients with known carcinoid syndrome
[c]) Lyphocheck® Control Urine II, Lot. No. 00200
[] Values in square brackets were not used for the determination of the correlation coefficient

Tab. 4. Determination of 5-hydroxy-3-indole acetic acid in urine and serum of three healthy donors and two patients with carcinoid syndrome.

	5-Hydroxy-3-indole acetic acid concentration (μmol/l)		Serotonin concentration[a]) (μmol/l)
	Urine	Serum	Serum
S. H.	18.8	0.72	0.64
I. K.	20.9	0.81	0.46
A. H.	14.6	0.3	0.85
Sample 24[b])	581.0	5.23	6.2
Sample 25	371.0	5.75	5.05

[a]) Determined by a commercially available radioimmunoassay (DDV Diagnostika, Marburg, F. R. G.)
[b]) See Table 3.

Discussion

We determined 5-hydroxy-3-indole acetic acid by radioimmunoassay, because this method is faster, more sensitive and more specific than previously described methods (1−9). The determination of the methyl ester of 5-hydroxy-3-indole acetic acid has several additional advantages: firstly, esterification prior to radioimmunoassay strictly limits the number of possible cross-reacting compounds to carboxylic acids; secondly, endogenous N-acetylserotonin, a minor metabolite of 5-hydroxytryptamine (14), which could falsify the assay is detectable by direct comparison

of esterified and normal urine. Nevertheless, neither N-acetylserotonin nor any other cross-reacting unknown substance was found in normal and pathological urines under assay conditions.

The present investigation shows that the radioimmunoassay of esterified 5-hydroxy-3-indole acetic acid is highly sensitive for the analysis of 5-hydroxy-3-indole acetic acid in a variety of urine samples as well as in serum. The good coefficients of variation exhibited by the method should facilitate the examination and comparison of groups that differ only slightly from one another with respect to 5-hydroxy-3-indole acetic acid levels.

References

1. Oates, J. A. & Butler, T. C. (1967) Adv. Pharmacol. *5*, 109–114.
2. Udenfriend, S., Weissbach, H. & Clark, C. T. (1955) J. Biol. Chem. *215*, 337–344.
3. Udenfriend, S., Titus, E. & Weissbach, H. (1955) J. Biol. Chem. *216*, 499–505.
4. Young, D. S., Thomas, D. W., Friedman, R. B. & Pestaner, L. C. (1972) Clin. Chem. *18*, 1268–1276.
5. Anderson, G. M. & Purdy, W. C. (1979) Anal. Chem. *51*, 283–289.
6. Shihabi, Z. K. & Scaro, J. (1980) Clin. Chem. *26*, 907–909.
7. Ranadive, N. S. & Sehon, A. H. (1967) Can. J. Biochem. *45*, 1689–1699.
8. Ranadive, N. S. & Sehon, A. H. (1967) Can. J. Biochem. *45*, 1701–1710.
9. Delaage, M. A. & Puizillout, J. J. (1981) J. Physiol. *77*, 339–347.
10. Manz, B., Kosfeld, H., Harbauer, G., Grill, H.-J. & Pollow, K. (1985) J. Clin. Chem. Clin. Biochem. *23*, 657–662.
11. Manz, B., Schüler, G. & Pollow, K. (1986) Acta Endocrinol. Suppl. *274*, vol. *111*, p. 235.
12. Manz, B., Grill, H.-J., Köhler, I., Heubner, A. & Pollow, K. (1982) Eur. J. Biochem. *128*, 249–255.
13. Al-Shawi, A., Mohammed-Ali, S., Houts, T., Hodgkinson, S., Nargessi, R. D. & Landon, J. (1981) The Ligand Quarterly *4*, 43–51.
14. Rollay, M. D. (1983) Methods for measuring pineal hormones (Reiter, R. J., ed.) CRC Press, Boca Raton, Florida, pp. 274–298.

Dr. Bernhard Manz
Abteilung für Experimentelle Endokrinologie
Johannes Gutenberg-Universität
Langenbeckstr. 1
D-6500 Mainz